ON MUSCLE REGENERATION IN THE LIMBS OF PLETHEDON.

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SPALLANZANI (1768) and Bonnet (1777) showed that a salamander whose limbs have been cut off has the power to regenerate new ones. This discovery has been confirmed by later writers, and although some histological work has been done, yet the method of regeneration of the muscle bundles has not been worked out. There are several possibilities: first, the old fibers might break down at the cut ends and the new ones develop from the indifferent tissue so formed, each old muscle thus completing itself independently. Or, the cut muscles might degenerate along their entire length, and new ones take their place; or some of the old muscles might degenerate, new ones being formed from this tissue, while some fibers might break up into smaller new fibers. An attempt has been made in this work, not so much to follow the origin of the cells in detail as to discover the general processes taking place in the leg that lead to the formation of the new muscles. The regenerating limbs of *Plethedon cinereus* were used. They were studied by means of serial sections.

In addition to this histological study, I have also experimented on a number of American urodeles in order to see in which ones regeneration of the limbs takes place. For this purpose a number of the commoner forms have been studied, and in connection with these results a statement is given of the previous observations on European forms.

I.

Method. — One of the anterior limbs of *Plethedon cinereus* was removed halfway between elbow and hand. The regenerating limbs were put up at intervals varying from four days

to two weeks. They were fixed in corrosive acetic, hardened for two or three days in 95% alcohol, and then decalcified for from six to eight days in a nitric acid solution (HNO_3 sp. gr. 1.42, 2 vols. + H_2O, 98 vols.) which was changed daily. They were finally hardened again for three days in 95% alcohol, embedded and cut. Some limbs were stained *in toto* with borax carmine, but the best results were obtained by the method used by Byrnes ('82), *viz.*, staining on the slide in Delafield's haematoxylin, followed by a wash of picric acid in absolute alcohol. This latter method differentiates the muscle substance very clearly.

Eleven stages were preserved at the following intervals:

	Time of Operation.	Time of Killing.	Age of Stump.
1.	May 4, 1900.[1]	May 14, 1900.	7 days.
2.	" " "	" 18, "	11 "
3.	" " "	" 29, "	22 "
4.	Oct. 22, 1899.	Nov. 20, 1899.	29 "
5.	" " "	Dec. 5, "	44 "
6.	" " "	" 15, "	54 "
7.	" " "	" 15, "	54 "
8.	" " "	Jan. 5, "	75 "
9.	" " "	" 5, "	75 "
10.	" " "	" 20, "	90 "
11.	" " "	March 22, "	151 "

Transverse sections of this series were cut. Nos. 1, 2, and 3 were stained with haematoxylin and picric acid; 4, 5, and 6 with borax carmine; 7 with borax carmine and picric acid; 8 and 9 with Biondi-Ehrlich solution;[2] 10 and 11 with haematoxylin and picric acic. In addition, normal limbs were cut and stained in a similar manner and used for comparison.

Results. — For convenience in description I shall consider the sectioned limb as made up of three Regions: I, that between the cut and the elbow; II, the Region just above the cut; and III, the growing end. In the earliest stages Region III does not, of course, exist.

[1] It will be noticed that stages 1, 2, and 3 were preserved later in the year, but observations will be described in the above order.

[2] This stain was not successful, and the stages were replaced by one stained with haematoxylin and picric acid.

In the first of the series of transverse sections changes in the cut muscles are already noticeable, the most striking being the increase in the number of nuclei, especially in the outer fibers of the limb. This increase can be seen in Region I as far up as the origin of the muscles at the elbow. In the outer fibers the muscle tissue is becoming thinner and disappearing, and while the outlines of fibers and bundles are not lost, they are much less clear than in the normal limb. The inner bundles, however, are but little affected, and extend unbroken to the cut end, which is at this time not yet entirely covered by ectoderm. No mitosis is seen in this section.

In stage 2 the changes are more marked. There has been a continued increase in the number of nuclei in Regions I and II, the outlines of the outer fibers and bundles are lost, while the muscle substance has disappeared except for disintegrating fragments here and there, contrasting sharply with the thin cytoplasm of the neighboring cells. The inner fibers still extend to the end of the limb, which is now covered entirely by several layers of ectoderm. In the neighborhood of the cut three or four mitotic figures are to be found. In the third stage Region III begins to appear as a small knob of undifferentiated tissue behind the cap of ectoderm. In this knob and for a short distance above it among the outer cells karyokinesis is not uncommon.

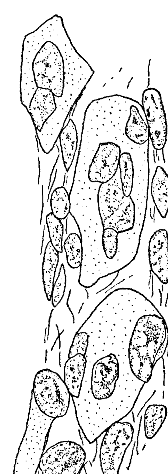

Fig. 1.

If we compare the following stage (4) with the normal limb, the principal changes that have taken place will be very clearly brought out. In Region I the increase in number of nuclei is very great. Even as far as the elbow two to four nuclei may be found in a section of a single fiber, often crowded together so as almost to fill it (Fig. 1). Many nuclei are also scattered between the fibers. Below the elbow the number of nuclei increases, the outlines of the outer fibers are completely lost, and the outer half of the limb, which is normally solid muscle, is seen to be made up of a dense mass of nuclei surrounded by loose protoplasmic substance, with here and there

clumps of disintegrating muscle tissue. The outlines of the inner fibers are somewhat less distinct than in the preceding stage, and some of the bundles seem to be splitting up. This condition is represented in Fig. 2. Most significant is the fact that at no stage is any karyokinesis found among the muscle fibers, although the increase in the number of nuclei is enormous. Passing through Region II of this stage, the number of old fibers decreases and the scattered nuclei increase, until at the

FIG. 2.

plane where the cut was made all old fibers disappear and we reach Region III, which is made up entirely of closely crowded nuclei, each surrounded by a small amount of protoplasm. Karyokinesis is first seen in II, a short distance above the cut, among the outer cells, *never in the muscle fibers*, and the number of dividing cells increases toward the growing tip until it becomes quite large.

In the fifth stage a further difference is to be noted. We find in Region I, in the inner part of the limb, the old muscle

fibers, often with several nuclei, and broken frequently into quite small fragments. Outside these are numerous nuclei, as before, but now surrounded by very distinct muscle tissue (Fig. 3). This tissue has formed often about several nuclei in a group, and has no distinct walls; there seem to be as yet no distinct muscle fibers. But the line of separation between the old muscle fibers and the new tissue is distinct, and a few old fibers can be traced to the region of the cut, although at that level the greatest part of the tissue is new. New muscle substance has appeared about the nuclei for a short distance below the cut; but it decreases as we pass down, until we find only crowded nuclei and thin protoplasm. In this region, as before, numerous karyokineses are seen.

FIG. 3.

In Region I of the sixth stage all the muscle fibers are small and the definite line between the old and new is lost. The majority of the fibers contain in cross-section but one nucleus, though some may contain two or three, and in general the smallest fibers and most nuclei are on the outer side of the limb. This is especially noticeable in Region II, where the outer (new) fibers are exceedingly small. The muscle tissue decreases in amount as we pass to Region III, until it is all lost. Cells dividing by karyokinesis appear at this level.

Stage 7, though of the same age as the preceding, is somewhat further differentiated, and in this the new fibers are more rounded and have assumed a more characteristic form (Fig. 4). A comparison with the normal shows smaller fibers

and great excess of nuclei, there being often two or three to one fiber and many outside the fibers.

The later stages need not be described in detail. As the limb grows longer the formation of new muscle tissue progresses farther down toward the tip, the new fibers being always small and containing several nuclei. The number of nuclei outside the fibers decreases, until in stage 11 the muscles look quite normal, and the number of nuclei is excessive only in the region of the foot, which is at this time

FIG. 4.

clearly differentiated. Karyokinesis is found, I believe, without exception, near the growing end, never in the upper regions.

The first appearance of any definite grouping of cells appears in stage 5, where the arrangement into bundles is foreshadowed. As the fibers form, the division into bundles becomes more distinct, until in stage 11 they are all differentiated as far down as the foot, and here we can see by the arrangement of nuclei where the bundles are to be.

In the process of regeneration described above there are certain things to which I wish to direct especial attention. In

the first place there is a great increase in the number of nuclei *within the old fibers*, but in no case is any karyokinesis found there. This degenerative process in the old fibers must therefore take place by direct division of the nuclei. Instances of this division are shown in Figs. 5 and 6. To this division and to the disintegration of some of the old fibers is due the enormous accumulation of nuclei in the outer part of the limb (Fig. 2). The cells so formed then begin to divide by karyokinesis in the region of the cut, and thus a further increase in their number takes place. In these outer cells new muscle tissue forms and the new fibers are built up. A certain

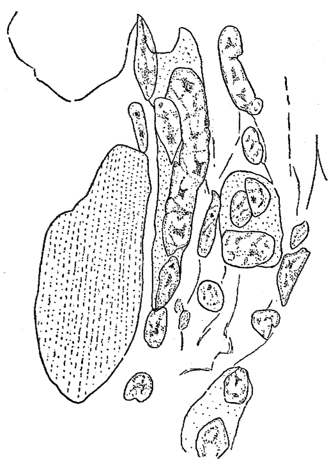

Fig. 5.

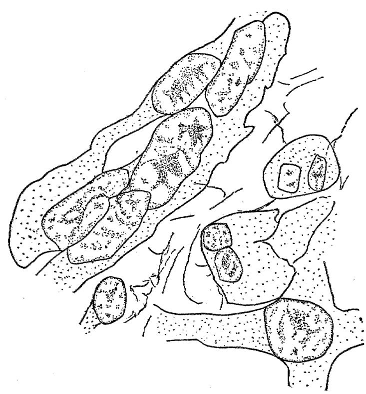

Fig. 6.

number of the old fibers remain in the middle of the limb, and in these the muscle tissue never disintegrates, though it splits longitudinally.

Again, as at any one level the number of nuclei far exceeds the number of fibers in a normal muscle, a great number of them must, between the early stages and the fully formed limb, either degenerate or be transported (*cf.* Figs. 3 and 4). That this is so is easily seen, for two reasons: (1) when the new fibers form, at a given level several nuclei are often included in one fiber; when the limb is full-grown there is only one; (2) among the newly formed fibers, but *between* them, are many scattered nuclei; the majority of these disappear in later stages.

One further point should be mentioned. In stage 5 the line of distinction between new and old fibers is clear, owing to their difference in size. In stage 7 this distinction has disappeared. This is due not only to an increase in the size of the new fibers, but to a decrease in the old. This decrease is, I believe, due to the longitudinal splitting of such of the old fibers as are left (Fig. 7. *a* and *b*).

Beside the stages described above, four others were preserved and cut longitudinally. This was a somewhat difficult operation, for the new part forms at an angle with the upper arm, and it is hard to orient the piece in such a way as to insure true longitudinal cutting of the muscle fibers. The material was preserved at the following intervals:

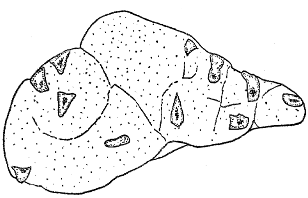

FIG. 7 *a*.

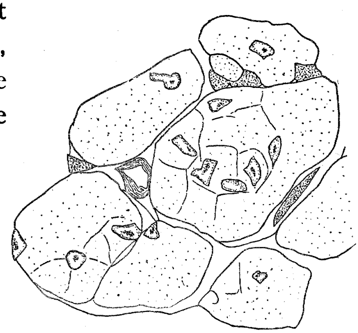

FIG. 7 *b*.

Time of Operation.	Time of Killing.	Age of Stump.
1. Jan. 29, 1900.	Feb. 2, 1900.	4 days.
2. " " "	" 12, "	14 "
3. " " "	" 19, "	21 "
4. " " "	" 28, "	30 "

The first of these shows no transformation at the cut ends of the muscles, but the ectoderm has closed in over the wound. In the second there is a considerable increase in the thickness of the ectoderm, and under it a collection of scattered nuclei, exactly similar to the tissue in the growing end of later stages. As the limb grows in length this tissue increases in amount and a number of mitoses are seen in it. Degeneration of the old fibers is distinctly noticeable in stage 3, and fibers are found, as before, filled with nuclei, but there is no karyokinesis in any muscle fiber.

SUMMARY.

The main changes that take place in the muscles of *Plethedon cinereus* during the process of regeneration are as follows :

1 (*a*) In the cut muscles the nuclei divide directly and in the outer bundles the fibers disintegrate, leaving masses of nuclei with a small amount of cytoplasm. (*b*) Some of the cells so formed later divide mitotically, and by them new muscle substance is laid down. (*c*.) As the number of nuclei is, however, far in excess of the normal number of muscle fibers, many nuclei must degenerate or be transported.

2. The fibers of the inner bundles do not disintegrate, but split longitudinally, giving rise to smaller fibers, which are soon indistinguishable from those formed as described in 1.

3. The arrangement into muscle bundles first becomes clear at the end of about six weeks.

4. There is no change in the muscles of the upper arm.

II.

The following is a brief summary of the main observations that have been made on the power of regeneration of the limbs in European species of salamanders.

Spallanzani (8), in 1768, published a number of observations on aquatic salamanders, presumably species of Triton. In these he found that any or all of the limbs will regenerate, no matter what the species, size, or age of the animal, the larger ones regenerating more slowly than the smaller forms. Regeneration will take place, he maintained, even when the limbs are disarticulated.

Bonnet, 1777 (2), confirmed Spallanzani's observations, finding that in *Triton cristatus* the hands and fingers will regenerate.

Von Siebold (7), in 1828, recorded abnormal regeneration of the fingers of *Triton cristatus.*

Higginbottom, in 1847 (4, p. 29), observed that in Triton the limbs will regenerate at a temperature of from 48° to 57° F.

Philippeaux (6), in 1866, was the first to prove conclusively that limbs when disarticulated will not regenerate. His experiments were made on *Triton cristatus* and Axolotl.

Dumeril (3), in his paper of 1867, in the course of other observations, notes the fact that in an Axolotl the two anterior limbs regenerated after injury.

Wiedersheim, 1875 (1, p. 95), found that the toes of *Triton cristatus* will regenerate, while there is no regeneration of the limbs in Proteus and *Siren lacertina.*

Erber, 1876 (4, p. 34), notes regeneration of the feet of *Siren lacertina.*

Goette, 1879 (5), records the regeneration of a leg of Proteus after a year and a half. Regeneration also occurs in Amphiuma and Siren, in *Triton cristatus*, *T. taeniatus*, and their larvae.

Weismann (9), in The Germ Plasm, 1893, says that the limbs of *Salamandra* regenerate, while in *Triton marmoratus* regeneration is slight or absent.

Barfurth (1), in 1894, reports regeneration of the feet and digits of *Triton taeniatus* and *Siredon pisciformis.* This regeneration is normal or abnormal according to the plane and method of the injury.

I have experimented on the following forms, removing a fore foot and a hind foot from different individuals of each species: *Plethedon cinereus*, *Spelerpes ruber*, *S. guttolineatus*, *Desmognathus ochrophaea*, *Manculus quadridigitatus*, *Amblystoma opacum*, *Diemyctylus viridescens*, *Amphiuma means*, and *Necturus maculatus.* Of these, all have regenerated.[1] The regeneration in Spelerpes, Desmognathus, Manculus, and Amblystoma was comparatively rapid, and new limbs were well formed in four months, though they were somewhat smaller than the old limbs. Diemyctylus was slower in reaction, while the first Necturus to show a distinct regenerated stump did so only after eight months. Other individuals of the same species showed no regeneration even at that time.

I desire to thank Professor T. H. Morgan, under whose direction this work was undertaken, for his kindly assistance during its progress.

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[1] *Amphiuma means* was observed for only eleven weeks. At that time regeneration was slight. The regenerated stump was not sectioned.

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